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TITLE: Investigating the Role of Indoleamine 2,3-dioxygenase (IDO) in Breast Cancer Metastasis

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14. ABSTRACT

Indoleamine 2,3-dioxygenase (IDO1) is a tryptophan catabolizing enzyme known to support primary tumor outgrowth through immune suppression though there is little data addressing its role in metastasis, an important aspect of tumor malignancy. Tumors formed by orthotopic engraftment of the malignant 4T1 breast carcinoma cell line exhibit metastatic spread to organs similar to that seen in human breast cancer with pulmonary metastases being the primary cause of mortality in this model. To determine the role of IDO1 in breast cancer metastasis, we have utilized IDO1 knockout (IDO1-/-) mice to directly study the impact of IDO1 loss in the host. While primary tumors in IDO1-/- mice exhibited a similar growth rate to that observed in the wild-type (WT) control, survival was significantly increased in the IDO1-/- mice. Further analysis of IDO1-/- mice showed approximately 10-fold less metastatic burden in the lungs of these mice. Serum isolated from IDO1-/- and WT controls showed similar levels of metastatic cells indicating that the reduced metastatic spread is not due to decreased tumor cell migration but rather to the reduced ability to establish tumor metastases. Evaluation of the tumor microenvironment showed that IDO1 protein and activity in WT mice directly correlate to metastatic burden. Higher levels of MCP1 and IL6 were observed in WT mice compared to IDO1-/-. The immune cell profile in these two populations also differs, leading us to conclude that IDO1 expression in normal lung tissue influences the immune response and supports the development of metastases.

15. SUBJECT TERMS
Indoleamine 2,3-dioxygenase, pulmonary metastasis, microenvironment, immune response

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INTRODUCTION:

Treatment of cancer commonly entails surgical resection followed by chemotherapy and radiotherapy, a regimen that results in variable degrees of long-term success. This is in part due to the ability of tumor cells to escape these methods of treatment and restore primary tumor growth and more importantly distant metastasis. The majority of cancer related deaths are due to the development of metastatic disease as opposed to primary tumor burden. In human breast cancer, the lungs are the primary site of metastasis followed by bone (1,2). Evidence of metastasis in breast cancer patients is considered a strong negative prognostic factor. Therefore advances in treatment to reduce metastasis will greatly improve survival in breast cancer.

The finding that there is a synergistic benefit to combining chemotherapy with the indoleamine-2,3-dioxygenase (IDO1) inhibitor 1-methyl-tryptophan (1MT) in preclinical mouse models of breast cancer, (3) suggests a promising new therapeutic approach. IDO1 is the rate-limiting factor in tryptophan catabolism; however, it is not involved in dietary catabolism in the liver, leading researchers to determine an alternative role for this enzyme. The seminal demonstration that 1MT could elicit MHC-restricted T cell-mediated rejection of allogeneic mouse concepti (4,5) established a role for IDO1 in mediating immune tolerance. Studies have also revealed a pathophysiological link between IDO1 and cancer, with increased levels of IDO1 activity being associated with a variety of different tumors (6,7). The therapeutic potential of targeting IDO1 in conjunction with chemotherapy has been demonstrated in the MMTV-Neu mouse model of breast cancer. The effects of 1MT were found to be greatly enhanced when given in conjunction with the commonly used chemotherapeutic agent paclitaxel (3). Depletion of either CD4+ or CD8+ T-cells in these mice abolished the benefit provided by 1MT indicating the importance of T cell immunity in the antitumor response.

The immunosuppressive function of IDO1 manifests in several manners. Collectively, IDO1 and its metabolites can directly suppress T cells (17-20) and NK cells (21) as well as enhance local Tregs (22). While there are no currently published studies, the protumorigenic capabilities of myeloid derived suppressor cells (MDSCs)(13-16) suggest that this population may also be affected by IDO1. Furthermore, IDO1 is produced in response to IFN-γ, an important cytokine modulator of inflammation. It is therefore reasonable to hypothesize that IDO1 not only regulates immune cells but may be regulated by or may regulate cytokine production in the host resulting in a protumorigenic microenvironment.

To address these questions, we have characterized tumorigenesis in IDO1-/- mice to allow for the study of IDO1 in metastasis. Using an immune competent model we focused on the role of IDO1 in the immune response to tumors. We have selected the highly metastatic 4T1 breast cancer model which progresses similarly to human breast cancer (10,12). Using this model, we were able to compare the metastatic sites of IDO1-/- and WT mice and evaluate the importance of IDO1 in metastatic outgrowth. Our studies in this model demonstrate that the loss of IDO1 improves survival due to reduced pulmonary metastasis and furthermore, this occurs through the reduced immunosuppressive response of the host.

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BODY:

Prior to commencement of all animal related experiments, IACUC approval to conduct the proposed experiments was obtained. In our **first specific aim,** we proposed to evaluate IDO1 activity in the lungs of 4T1 tumor-bearing mice and determine the source of its expression. 4T1 cells were orthotopically injected into the mammary fatpad of BALB/c and IDO1-/- mice. After 2 weeks, palpable tumors were observed and two perpendicular measurements were taken at weekly intervals over a six week period to evaluate primary tumor growth. Primary tumors in IDO1-/- mice exhibited a similar growth rate to that observed in the wild-type control (**Fig. 1A**). However, survival was significantly increased in the IDO1-/- mice by an average of 22 days (**Fig. 1B**). The 4T1 model is a well characterized system to replicate stage IV breast cancer due to the spontaneous metastasis to lungs, liver, lymph node and brain. The highly metastatic nature of 4T1 suggested that the difference in survival, while not related to primary tumor burden, may be a result of disproportionate metastatic burden. Therefore, lung, liver and brain were harvested along with blood and analyzed by the clonogenic assay for metastatic tumors. Metastatic colonies in the lung showed that there was less metastatic burden in the lungs of IDO1-/- mice by approximately 10-fold (**Fig. 1C**). Serum isolated from IDO1-/- and wild-type controls showed similar levels of metastatic cells indicating that the reduced metastatic spread is not due to decreased tumor cell migration but rather to the reduced ability to establish tumor metastases (**Fig. 1C**). The observation in lung was further confirmed using both India Ink re-inflation of the lungs and microCT (**Fig. 1D**). Injection of India Ink into the lungs allows normal lung tissue to absorb the stain causing metastatic regions to be visualized as white nodules. Similarly, microCT shows normal lung as a dark region while the significantly denser metastatic regions appear white, further confirming that the improved survival of IDO1-/- mice is due to reduced pulmonary metastasis. Metastatic colonies in the liver and brain did not exceed 10 per organ and had no statistical difference between WT and IDO1-/- mice (**Fig. 1E-F**).

As proposed in the statement of work, these experiments were completed in the first year and a repeat performed in the second year to confirm the original observations. The second study showed the same pattern of reduced metastatic burden and increased survival in IDO1-/- mice providing a final n-value of greater than 20 mice per group.

The reduction in metastasis observed in the IDO1-/- mice suggests that IDO1 is pro-metastatic. We therefore investigated the presence of IDO1 protein in the microenvironment of the lung. Time points were collected at 1 week intervals between 2-6 weeks for WT and 2-8 weeks for IDO1-/- mice. Baseline levels were obtained from non-tumor-bearing mice. In WT mice early metastases are observed at approximately 3-4 weeks. By 5 weeks there is a substantial metastatic burden, leading to a lethal burden in week 6. By comparison, these timepoints are shifted two weeks later for IDO1-/- mice requiring IDO1-/- timepoints to be extended to 8 weeks. No data at 7 and 8 weeks is available for WT mice as they do not survive past 6 weeks. Protein IDO1 was measured by immunoprecipitating with αIDO1 and probing a Western blot with another IDO1 antibody. As expected, IDO1-/- mice do not express any IDO1 protein, however, WT mice have greater levels of IDO1 as metastatic burden increases (**Fig. 2A**). Using LC/MS/MS to measure the presence of kynurenine, a product of tryptophan catabolism by IDO1, we were able to demonstrate that the IDO1 protein in the lung microenvironment is also

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functionally active and that the level of kynurenine increases directly in relation to the protein (**Fig. 2B**). This further supports the importance of IDO1 in the lungs for optimal conditions of metastatic outgrowth. As with the previous figure, these data were collected in a second cohort of mice in year 2 to increase the n-value to greater than 10 mice per group.
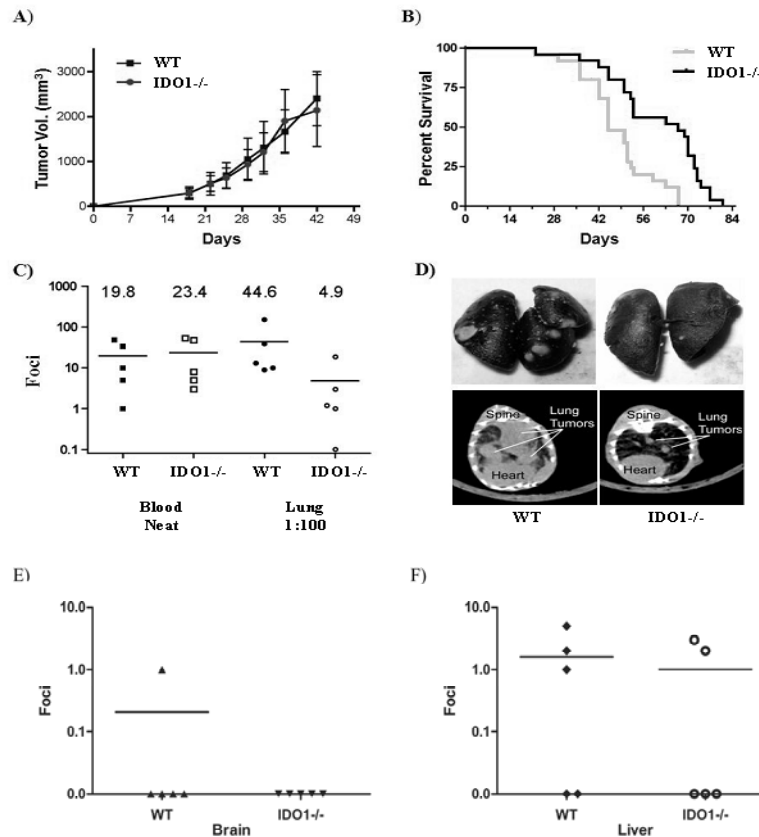
Figure 1: IDO1 is critical in pulmonary metastatic outgrowth, not primary tumor growth. Mice were injected orthotopically with 4T1 cells (A-F). (A) Primary tumor burden was measured in WT and IDO1-/- mice showing no difference in growth rate. Data are representative of 3 experiments. (B) Survival studies were plotted as a percent survival over time. Significance was determined by a 2-group log-rank test (Mantel-Haenszel test). (Increased median survival = 22 days, P < 0.0001) Data representative of two independent experiments. (C) Number of metastatic colonies determined by clonogenic assay at 6 weeks following 4T1 engraftment for individual mice are plotted with a mean value for each group and SEM. Statistical analysis performed using the 2-tailed Mann-Whitney test. Data are pooled from two independent experiments. (D) Visualization of pulmonary metastases by India Ink (upper) and microCT (lower). (E-F) Brain (E) and liver (F) metastasis measured by clonogenic assay at 6 weeks following 4T1 engraftment.
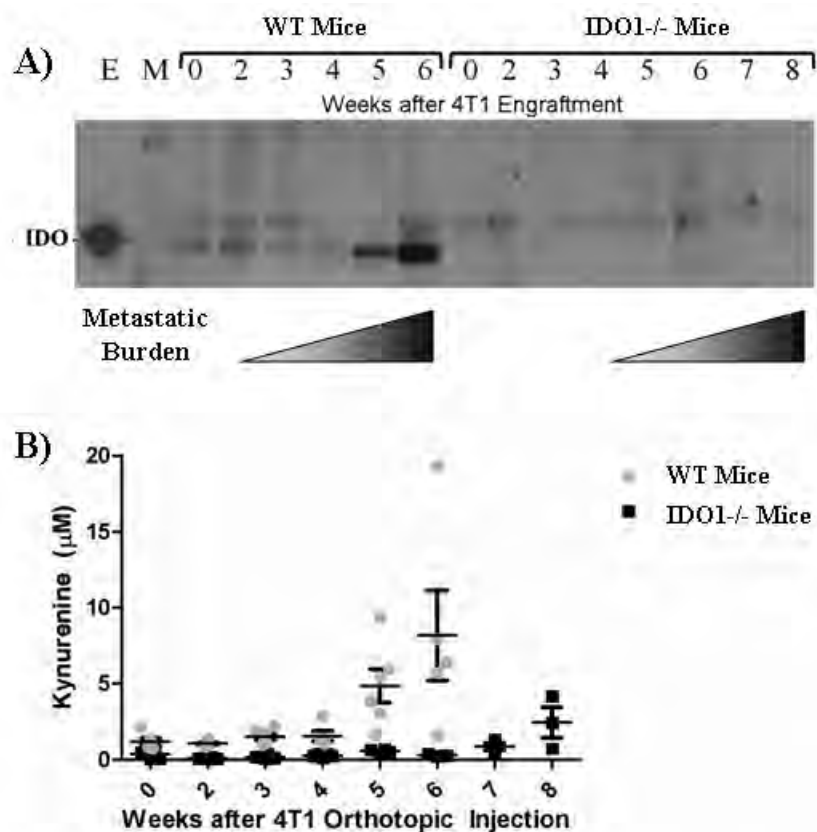
Figure 2: IDO1 increases with metastatic burden. (A) Immunoprecipitation of lung homogenate using αIDO1 was run on a Western blot and probed with αIDO1. (B) Kynurenine levels present in homogenized lung from WT and IDO1-/- mice were measured by LC/MS/MS.

Current roadblocks remain in the difficulty of obtaining an IDO1 antibody suitable for immunohistochemistry. Until recently, existing antibodies have not been used on IDO1-/- mice and therefore no publications of IDO1 immunohistochemistry have this control. Our lab and others have observed high levels of non-specific staining in IDO1-/- samples, suggesting that these antibodies are not effective for IHC purposes. Currently we are collaborating with other researchers at LIMR to develop our own antibody to IDO1. To this end we have generated three antibodies that we are testing by immunohistochemistry. The continued focus on developing an IDO1 antibody remains, though we have completed the other components of Aim 1, including confirmation of the original data.

The **second specific aim** is designed to characterize the immune response in the lungs of 4T1 tumor-bearing mice. At the conclusion of last year, we were able to begin the bone marrow chimera test runs originally scheduled for Year 2. To effectively determine the reconstitution of the irradiated mice, we proposed the use of C.B-17 mice. C.B-17 mice are a congenic strain on a BALB/c background that carries the immunoglobulin heavy chain allele (Igh-1b) found on C57BL6 mice. This provides a marker to determine the level of reconstitution. Our pilot run has shown that we have greater than 99% successful reconstitution in our mice. Furthermore we found that the use of C.B-17 mice in place of BALB/c did not affect the rate of lung metastasis as determined by the number of circulating tumor cells and number of pulmonary metastases counted by the colony forming assay **(Fig. 3A)**.

Following the initial pilot experiment, we set up two bone marrow chimera experiments to measure the effects of the immune cells. The first experiment was set up as a clonogenic colony forming assay (**Fig. 3B**) and the second as a survival study (**Fig. 3C**). Both experiments resulted in greater metastasis susceptibility of mice with IDO1-/- marrow transplanted into an irradiated IDO1-/- mouse compared to irradiated WT mice receiving WT bone marrow. As these serve as our control groups and the data runs counter to our expected results, we are unable to interpret the experimental conditions of transplanting WT mice with IDO1-/- marrow and visa versa. We are currently investigating reasons for the difficulties with the control groups. Due to both experiments resulting in the same outcome, we must consider the possibility that IDO1-/- mice are more susceptible to metastasis as a result of irradiation.

While data from the bone marrow chimera experiments are inconclusive, we have proceeded to determine which immune cells may be involved in metastasis by focusing on the infiltrating immune cell profile. The profile was evaluated for differences between the WT and IDO1-/- 4T1 tumor-bearing mice by enzymatically dissociating lung tissue to form single cell suspensions for analysis by flow cytometry using the following panel of antibodies: αCD45, αCD4, αCD8, αCD3, αB220, αCD11c, αCD11b and αGr1. Lung samples were collected at 1 week intervals between 1-5 weeks following tumor engraftment. Between 2 and 3 weeks, the immunosuppressive MDSC population, identified here as CD11b+Gr1+ was greatly increased in the WT mice (**Fig. 4**). The data collected from these studies represent the percentage of positive cells from each immune cell population out of the entire CD45+ population. We can interpret the current data to indicate greater numbers of MDSCs in WT mice. These data parallel previous data in IL-1β knockout mice that showed the same delay in MDSC accumulation (24). This study went on to show that not only was there a delay but it reflected a defect in MDSC function. This may suggest additional experiments to evaluate the function of MDSCs in our model.

A)

B)

C)
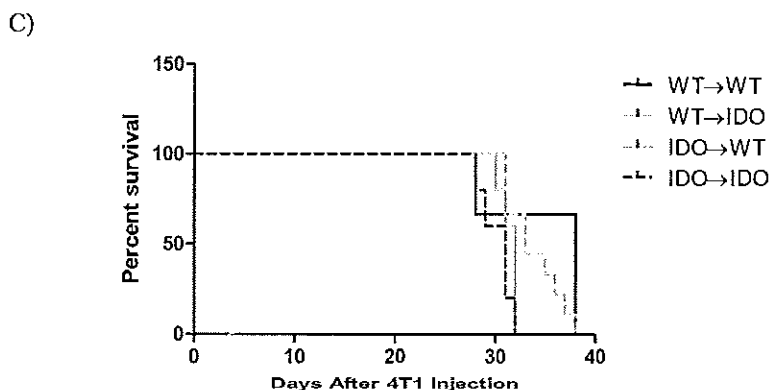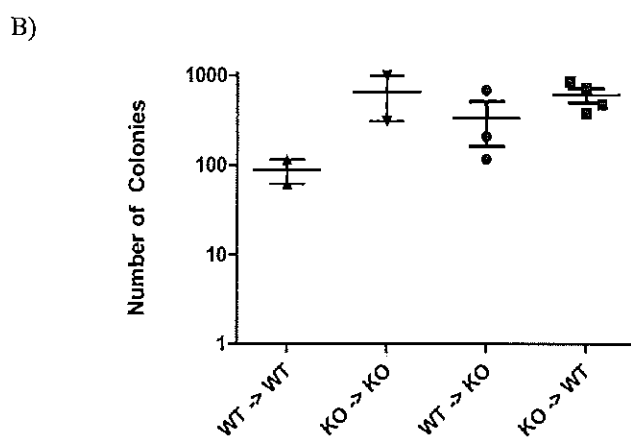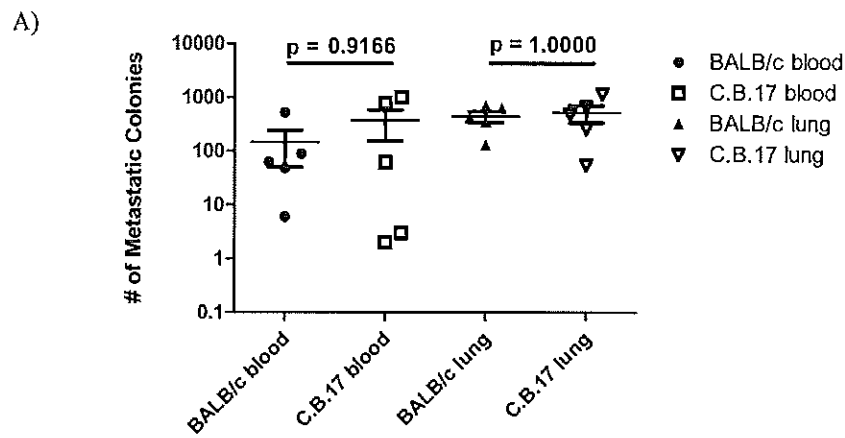
Figure 4: Bone Marrow Chimera Experiments. A) Lung tissue was homogenized and blood collected 6 weeks following orthotopic engraftment of 4T1 cells into BALB/c and C.B-17 mice. Colony forming assay was utilized to measure metastatic spread. BALB/c mice and C.B-17 mice have equal metastatic burden. B/C) Based on a small cohort of mice, a colony forming assay shows that control experiments run the opposite of expected, with KO to KO mice having greater metastatic burden compared to WT to WT mice. A small cohort in a survival assay had the same results, negating the use of either experiment.
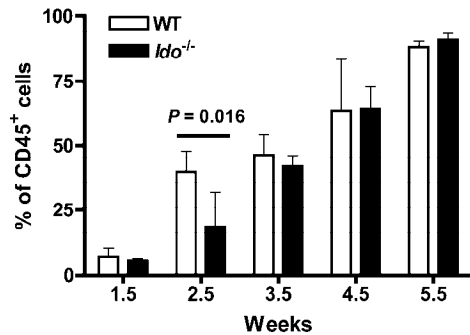
Figure 4: MDSCs are elevated early in WT mice. Whole lung tissues from WT and IDO1-/- mice at weekly time intervals were enzymatically digested to single cell suspensions for analysis by flow cytometry. Immune cells were selected by gating on CD45+ cells. Cells were further identified for the MDSC cell type by Gr1+CD11b+ expression.

The microenvironment of the lung affects the ability of 4T1 tumors to metastasize. Loss of IDO1 reduces tumor metastasis and is accompanied by a shift in the immune cell profile implicating the immune system in this suppression. Additionally, environmental cues may be altered such that tumor growth is not favored. Due to studies implicating a relationship between cytokines and IDO1, we hypothesized in **the third specific aim** that the cytokine profile in the lungs of tumor-bearing mice in the IDO1-/- versus the wild-type would be different.

Lung homogenates were evaluated using the Cytometric Bead Array (BD Biosciences) from samples collected at 1 week intervals between 2-5 weeks following tumor engraftment from groups of at least 4 mice each. This array uses amplified fluorescence detection by flow cytometry to detect soluble analytes to provide multiplexed data comparable to a classical ELISA. Initially, lung homogenates were analyzed for a set of cytokines that included IL-2, IL-4, IL-5 and IL-17 to determine differences in cytokines influencing immune cell maturation. Specifically these cytokines regulate divergence into Th1, Th2 or Th17 cell lineages. It is generally accepted that a Th1 environment suppresses tumor outgrowth while a Th2 environment promotes immune escape. The cytokine profile for Th1/Th2/Th17 showed no measurable levels of these cytokines above the limit of detection.

This prompted the use of a second panel of cytokines that reflect inflammation in the lung and include IL-6, IL-10, MCP-1, IFN-γ, TNF and IL-12p70. Samples were collected and analyzed similarly to the Th1/Th2/Th17 analysis. MCP-1 and IL-6, showed increased levels during tumor progression in WT mice (**Fig. 5A-B**). MCP-1 showed higher levels of expression in WT mice starting at 2 weeks and continuing through 6 weeks (**Fig. 5A**). This increase was on average two-fold greater than the IDO1-/- mice. While MCP-1 did increase in IDO1-/- mice, the response was less robust. Similar data for IL-6 was acquired with the difference that increased IL-6 was not observed until 4 weeks after orthotopic injection at which time there was a two-fold increase (**Fig. 5B**). At 5 weeks, IL-6 was on average 4-fold higher than the IDO1-/- mice that had only a marginal rise in IL-6 levels. These results collectively demonstrate that IDO1-/- mice have a suppressed inflammatory response to 4T1. Both TNF and IFN-γ showed equal elevations at each timepoint between WT and IDO1-/- starting at 3 weeks and continuing through 6 weeks (**Fig. 5C-D**). These cytokines are generalized responders to inflammation and may reflect a response to

the primary tumor burden or early tumor cell extravasation. There was no upregulation of IL-12p70 or IL-10 in either WT or IDO1-/- mice (**Fig. 5E-F**). We have completed the first run of the cytokine bead profiling and plan to proceed with repeating the data from the inflammatory profile. We will not proceed with the Th1/Th2/Th17 analysis as no significant measurements were obtained with the first run.

Based on our statement of work, we are projected to complete the proposed funded project in the three years of funding. We will continue to develop a method of detecting IDO1 for immunohistochemistry and will focus on the bone marrow chimeras and replicating the cytokine studies in the third year. While this model has similar metastatic progression as compared to human breast cancer, it would be beneficial to test our results in other mouse models. To this end, we have two proposed models to add to our studies. We propose to expand to a second model of metastatic breast cancer, E0771, in C57/BL6 mice. This would support our current experiments as well as expand the potential mouse models that can be bred onto this strain. The second proposed model is the PyVT mouse model of spontaneous breast cancer that is highly metastatic to the lung.
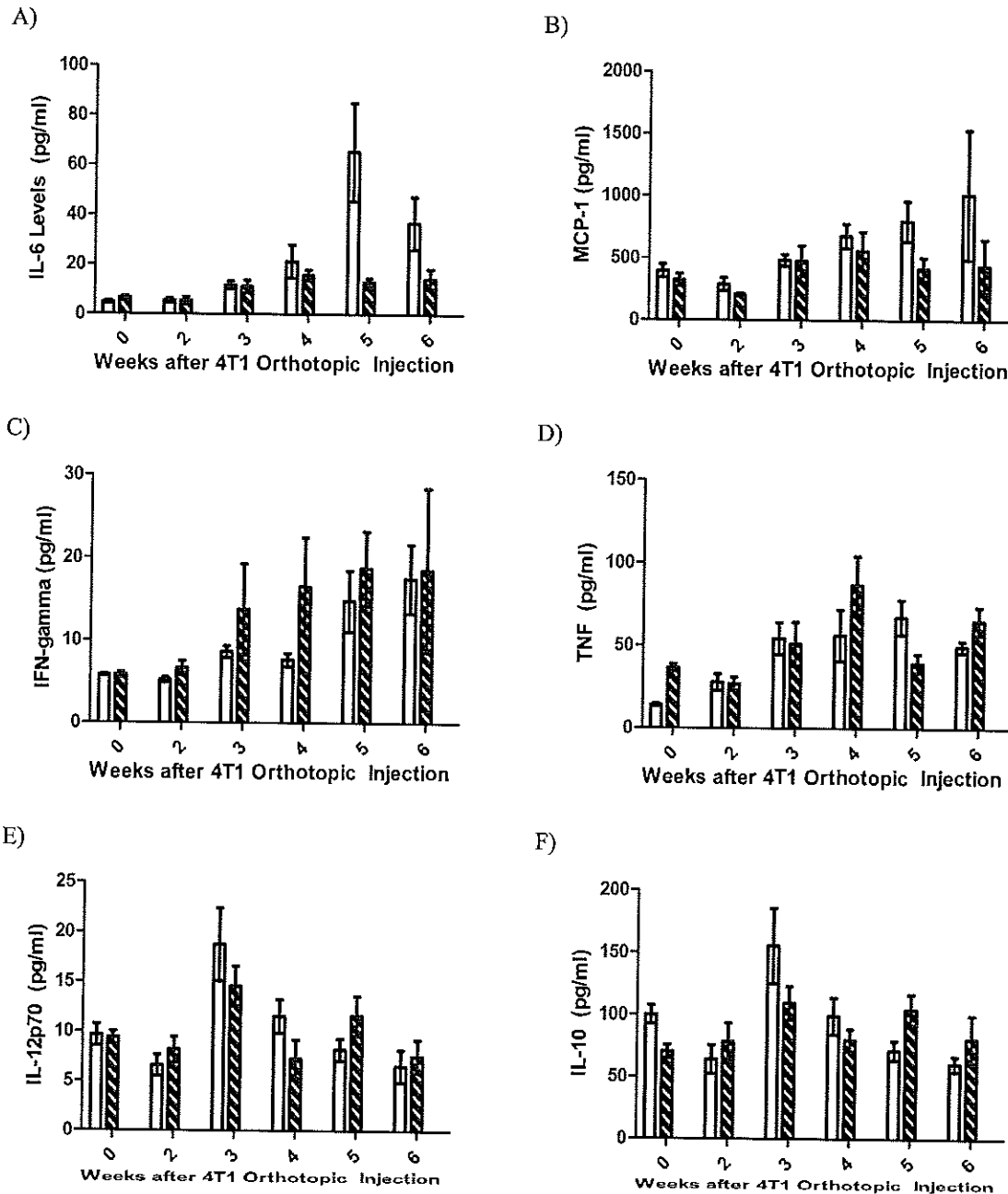
Figure 5: Metastasis induces cytokine induction. Homogenized lung tissue was analyzed for a panel of cytokines using the cytokine bead array. Cytokines are graphed as the average cytokine level in lung of five mice as a factor of time. Data shows mean and SEM. (A) IL-6 levels are induced at 5 weeks in WT mice compared to 8 weeks in IDO1-/- mice. (B) MCP-1 levels similarly increase at 5 weeks in WT mice but are maximally induced at 6 weeks. (C) IFN-γ levels are induced maximally at 5 weeks in WT and IDO1-/- mice. (D) TNF levels are induced at 2 weeks in both WT and IDO1-/- mice. (E-F) Neither IL-12p70 nor IL-10 showed significant changes in induction between WT and IDO1-/- mice.

KEY RESEARCH ACCOMPLISHMENTS:

- Found that the improved survival of IDO1-/- mice is due to reduced pulmonary metastasis
- Confirmed that expression of IDO1 protein is present in the metastatic site of the lung in the orthotopically engrafted 4T1 breast cancer model and correlated with activity as evidenced by increased kynurenine production
- Demonstrated that equal numbers of circulating tumor cells are observed in WT and IDO1-/- mice suggesting the effect of IDO1 loss and improved survival is due to a decreased adherence, extravasation or metastatic outgrowth
- Determined that the immunosuppressive MDSC population increases more rapidly in WT mice compared to IDO1-/-, a pattern previously seen in IL-1β knockout mice
- Completed a second set of experiments to confirm the observations previously stated
- Found measureable differences in inflammatory cytokine levels between IDO1-/- mice and the WT counterparts, particularly pertaining to the cytokines IL-6 and MCP-1

REPORTABLE OUTCOMES:

- Invited Speaker for Keystone Symposium on Molecular and Cellular Biology of Immune Escape in Cancer, Feb 7-12, 2010, Keystone Resort, Keystone, CO

- Travel Award/Conference Assistant for Keystone Symposium on Molecular and Cellular Biology of Immune Escape in Cancer, Feb 7-12, 2010, Keystone Resort, Keystone, CO

- Poster Presenter for Joint AACR Conference on Metastasis and the Tumor Microenvironment, Sept 12-15, 2010, Philadelphia, PA

- Chang, M.Y., **Smith, C.**, DuHadaway, J., Pyle, J., Boulden, J., Soler, A., Muller, A.J., Laury-Kleintop, L., Prendergast, G.C. 2011. Cardiac and gastrointestinal liabilities caused by deficiency in the immune modulatory enzyme indoleamine 2,3-dioxygenase. Cancer Biology & Therapy. *In Press.*

- Submitted book chapter. Prendergast, G.C., Metz, R., Chang, M.Y., **Smith, C.,** Ostrand-Rosenberg, S., Muller, A.J. 2011. Indoleamine 2,3-dioxygenase and tumor-associated macrophages: possible connection in cancer-associated inflammation and immune escape?

- **Smith, C.,** Chang, M.Y., Parker, K., Beury, D., DuHadaway, J., Flick, H., Boulden, J., Sutanto-Ward, E., Soler, A.P., Laury-Kleintop, L., Mandik-Nayak, L., Metz, R., Ostrand-Rosenberg, S., Prendergast, G.C., Muller, A.J. 2011. Indoleamine 2,3-dioxygenase is a nodal pathogenic driver of lung cancer and metastasis development. *In prep.*

CONCLUSION:

There are over 40,000 deaths each year in the US resulting from the metastatic spread of breast cancer. Based on data from our lab and others, an IND (investigational new drug) application for the IDO1 inhibitor D-1MT was approved last year and Phase I clinical trials with D-1MT have commenced. Breast cancer is identified in the clinical development plan as one of the high priority disease indications for evaluation in Phase IIA studies that will be used to determine the clinical scenarios best suited for the Phase II/III clinical development of this agent. Our recently published finding that D-1MT treatment in combination with cyclophosphamide chemotherapy significantly improved survival in mice bearing highly malignant 4T1 tumors (9) suggests for the first time that this approach may be applicable to metastatic disease as well.

Using the IDO1-knockout mouse strain, we have been able to genetically establish that IDO1 is important for supporting the development of pulmonary metastases from orthotopic 4T1 tumors. The core goal of the proposed project is to determine the underlying biological basis for this pro-metastatic effect of IDO1. The data produced by these studies will elucidate what we anticipate to be a novel mechanism of action through which IDO1 inhibitors can enhance the antitumor immune response achieved with cyclophosphamide chemotherapy, a frontline agent for the treatment of breast cancer patients. The results of these studies could have immediate bearing on how future clinical trials with IDO1 inhibitory compounds are designed and may lead to the development of more effective strategies for administering IDO1 inhibitors for the treatment of patients with metastatic breast cancer.

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